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14. ABSTRACT
We believe that the domestic laying hen has great potential as an animal model for ovarian cancer prevention research. Unlike other ovarian cancer models, which require experimental induction of ovarian tumors, chickens develop ovarian adenocarcinoma spontaneously, with an incidence ranging from 13 to 40 percent between four and six years of life. Few investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model remains to be validated and developed. As part of a prevention trial in chickens funded by the Department of Defense, we have accumulated 1400 chicken reproductive tracts including 140 with adenocarcinomas, and gathered valuable data regarding the natural history of these tumors. This provides the remarkable opportunity to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of the current proposal is to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, we will develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite to the widespread use of this animal model for ovarian cancer research.

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Ovarian Cancer, animal model, chemoprevention

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Introduction

Epithelial ovarian cancer is a highly lethal malignancy. It is the fourth to fifth leading cause of cancer deaths among women in the United States and causes 140,000 deaths annually in women worldwide. Despite intensive research efforts over the past decade directed towards improved detection and treatment, the long-term survival of women with ovarian cancer has only improved modestly. Progress in the fight against ovarian cancer has been hampered by a number of factors, including late diagnosis, molecular heterogeneity of tumors, absence of highly curative chemotherapy, and lack of a valid animal model.

We believe that development of effective chemopreventive agents for ovarian cancer represents our best hope for decreasing ovarian cancer mortality in the future. Based on our studies in primates and in the laboratory, we are convinced that the well-known protective effect of oral contraceptives against ovarian cancer is due, in large part, to the molecular biologic effects of progestins on the ovary. We have found that progestins differentially regulate TGF-beta expression and markedly activate the apoptosis pathway in the ovarian epithelium, making it more likely that cells that have incurred genetic damage will be eliminated, rather than develop into cancer. A number of other apoptosis-inducing agents also hold promise for preventing ovarian cancer, including retinoids. Ultimately, it is our goal to develop a preventive strategy using the best chemopreventive agents, either alone or in combination, in order to achieve maximum protection against ovarian cancer.

At this time, the lack of a valid ovarian cancer animal model is a major obstacle to ovarian cancer prevention research. In order to develop pharmacologic preventive strategies for ovarian cancer in a timely fashion, animal models that closely mimic human ovarian cancer are desperately needed. Human prevention trials are costly requiring large numbers of subjects and many years to complete. Development of an animal model for ovarian cancer prevention research would represent a significant breakthrough and lead to expedited evaluation of numerous candidate agents. Ideally, this would lead to rapid identification of a select number of agents, which have the greatest potential for ovarian cancer prevention and that can then be evaluated in human prevention trials.

We believe that the domestic laying chicken has great potential as an animal model for studying chemoprevention of ovarian cancer. Unlike other animal models for ovarian cancer, which generally require the experimental induction of ovarian tumors, the chicken develops ovarian cancer spontaneously. The domestic hen is the only animal with a high incidence of spontaneous ovarian adenocarcinoma, ranging from 13 to 40 percent between four and six years of life. Few investigators have taken advantage of the chicken to study ovarian cancer. Thus, the chicken ovarian cancer model remains to be validated and developed. As part of a chemoprevention grant awarded to us by the Department of Defense in 1998, we performed a two-year chemoprevention trial in the chicken designed to test the hypothesis that progestins confer chemopreventive effects against ovarian cancer. We have subsequently conducted a second prevention trial in the chicken, funded by the NIH, evaluating progestin and the retinoid 4-HPR as candidate preventives, and a third trial, funded by the Department of Defense is currently underway evaluating Vitamin D and progestin and ovarian cancer preventives. In addition to these trials, we have had the opportunity to examine different strains of hens at different ages, thus allowing examination of reproductive tract tumors that developed at different points during the lifetime of the hen.

As a consequence of the collective experience from the studies referred to above, we have collected a large set of chicken tumors, concomitant with valuable data regarding the natural history, development, and tissue distribution of these tumors. This has provided us with a remarkable opportunity to critically evaluate the chicken ovarian cancer animal model and determine its relevance to human ovarian cancer research. The aim of this grant proposal was to increase our understanding of the molecular and histologic features of chicken ovarian cancers. In addition, it was

our goal to develop a histologic classification for chicken ovarian cancers, which is a critically important prerequisite for the widespread use of this animal model for ovarian cancer research. For this proposal, we planned to characterize and develop the chicken ovarian cancer model by (1) analyzing the molecular and genetic features of chicken ovarian cancers, including alterations in the p53 tumor suppressor gene and the Her-2/neu and Ras oncogenes, (2) classify the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification for chicken ovarian adenocarcinomas, and comparing the molecular and histologic features of ovarian cancers that develop in chickens receiving synthetic progestins compared to untreated controls. The data that we have gathered has provided evidence that chicken ovarian cancers have genetic alterations and morphologic features similar to those identified in human ovarian carcinomas, thereby validating the chicken ovarian cancer model.

BODY:

Aim 1: To determine whether the genetic alterations that characterize ovarian cancers in women are also a feature of ovarian cancers in the domestic fowl.

A) Sequencing of p53 and ras

We have performed genetic analysis for mutations in the p53 and ras genes in 102 chicken reproductive tract cancers that developed during a large chemoprevention trial evaluating progestins and Vitamin D as ovarian cancer chemopreventives. The birds were 2 years of age when the two-year study commenced. They were given a calorically restricted diet, which inhibits ovulation and also ovarian cancer incidence. The histology of the tumors was reviewed by a poultry pathologist (Dr. Barnes). PCR was used to amplify the targets from tumor cDNA. The entire p53-coding region was screened for alterations using single stranded conformational analysis and direct sequencing of variant bands. Codons 12 and 13 of H- and K-ras were evaluated for mutations by direct sequencing. Results were compared to wild type sequences using NCBI Accession X13057 (p53) and XO3578 (H-ras). K-ras primers for sequencing were designed from the known sequence for M. gallopavo (turkey) Acc. No. X85754.

PCR and Sequencing Primers

Gene	Forward Primer	Reverse Primer	Size
Chicken H-Ras	TCAGCTGGAAGATGACCGAGT	TTGTGGGGTCGTACTCATCAA	114 bp
Turkey K-Ras	CGCCGGGCAGGTCTGCTAAAA	AGAGACAGGTTTCCCCATCAA	174 bp
P53-1	GCGGAGGAGATGGAACCATTG	GGGGAGTAAGTGCAGGTGACC	332 bp
P53-2	CCCATCCACGGAGGATTATGG	GGTCTCGTCGTCGTGGTAACG	342 bp
P53-3	GCCGTGGCCGTCTATAAGAAA	CGGAAGTTCTCCTCCTCGATC	396 bp
P53-4	CCTCACCATCCTTACACTGGA	GGTCCCTCCACCTTCACACGG	319 bp
P53-5	GCTGAACCCCGACAATGAGAT	GCGTGGCTAAAGGAAAAAGGG	222 bp

Clearly deleterious p53 mutations were found in 14/102 (13.7%) of chicken ovarian cancers. Thirteen of these mutations (93%) predict truncated protein products. There was one missense mutation (codon 368) that resulted in an amino acid substitution. All of the mutations were located between amino acids 101 and 336, which correspond to the DNA binding domains. An additional 14 chicken ovarian cancers had silent single nucleotide or intronic insertion polymorphisms. There was no relationship between the various treatment arms in the chemoprevention trials from which the birds were obtained and the presence of p53 mutations.

With regard to the ras gene, Chicken K-   ras shares a 90% and 99% homology to the human and turkey K-ras respectively. H- and K-ras mutations were not seen in codons 12 and 13 of H-ras or K-ras. Silent single nucleotide polymorphisms were noted within codon 39 of K-ras (G→C) with a frequency of 36%, and in codon 12 of H-ras in one case.

While the frequency of p53 mutation in this first study was less than that reported in human ovarian cancers, mutations in both species cluster in the critical DNA binding domains of the p53 gene. Additionally, despite being among the most common targets of mutations in various types of cancers, codons 12 and 13 of H-ras and K-ras are not mutated in chicken ovarian cancers, which is similar to human ovarian cancers that rarely exhibit ras mutations.

P53 Mutations found in 14/102 chicken ovarian cancers screened:

Bird	Sequence Change
1	G368C
2	451del147
3	451del147
4	393del57
5	649del3
6	803del122/C942A
7	750del51
8	803del202
9	803del122
10	803del122
11	800del122C927A
12	795del107/C927A
13	803del122/C942A
14	1073del15

From a second chemoprevention trial evaluating progestin and the retinoid 4-HPR, an additional 70 samples of chicken ovarian tumors were analyzed for H- and K-Ras and p53 mutations by direct sequencing. No large deletions were detected in these tumors, however one frameshift mutation and 4 deletions of one codon each (3 bp) were detected. All other mutations were missense mutations resulting in one or more amino acid alterations. In addition, 44/48 (92%) of the tumors completely sequenced had a mutation at nucleotide 277 resulting in a change of threonine to alanine at amino acid 72 (A277G). There were no noted mutations in the H-Ras gene in all 70 samples. Two mutations were noted in the K-Ras gene, both at the same locus. A substitution for T at position 176 for G results in the28th amino acid changing from phenylalanine to cysteine.

A complete list of the p53 mutations is found in the table below.

p53 Mutations in 24/70 Chicken Ovarian Tumors Screened

Bird	Sequence Change

1	G203A (S47N)
2	C178G (Q39E), 183 del 3 (del 40E)
3	C283T (P73S)
4	C251T (A62V)
5	T236C (L58P)
6	CT184AG, TG194GT, GC203AA, T236C, G247C, C251T
7	T236C (L58P)
8	A277C (T72P)
9	T236C (L58P)
10	178 del 3 (del 39Q)
11	263 ins C Truncates at 413 TAA; 118 aa instead of 368 aa, of which 50 aa differ
12	T236C (L58P)
13	T236C (L58P)
14	T236C (L58P), T242C (L60S)
15	T236C (L58P)
16	T242G (L60W)
17	178 del 3 (del 39Q)
18	T236C (L58P), T242C (L60S)
19	178 del 3 (del 39Q), T236C (L58P)
20	T236C (L58P)
21	T236C (L58P)
22	T236C (L58P)
23	T236C (L58P)
24	AG187GA (S42D)

An overview/summary of the mutation rate of this second sequencing study is shown below.

Summary of Sequencing Results for 2nd Study

	p53	K-Ras	H-Ras	Total	% Mutated
Control	5	1	0	17 (24%)	6/17 (35%)
4HPR Tx	11	0	0	28 (40%)	11/28 (39%)
Levo Tx	9	1	0	25 (36%)	10/25 (40%)
Total	25 (36%)	2 (3%)	0	70 (100%)	27/70 (39%)

Unlike the first sequencing foray which detected several deletion mutations within the DNA binding domain, this second group of ovarian chicken tumors has the bulk of its mutations in either the transactivation domain (aa 1-50) or the proline rich region (aa 63-97).

The p53 tumor suppressor gene is mutationally inactivated in some chicken ovarian cancers. Amino acid substitution mutations has a relatively high frequency in chicken tumors as well, although the effects of these mutations on folding and/or function are perhaps more subtle. The mutations noted here are primarily in the proline rich and DNA binding domains of the p53 protein, similar in location to where mutations are most frequently mapped in human ovarian cancers.

It is important to note that there is published evidence that the proportion of human ovarian cancers with p53 mutations increases commensurate with the number of lifetime ovulatory

events in women. [Schildkraut, JNCI 89: 932-   8, 1997] That is, the process of ovulation with repeated cycles of rupture and then repair of the ovarian epithelium probably increases the number of proliferative events, leading to more alterations in p53 in the tumors that subsequently develop. In our first chemoprevention trial, in which the incidence of p53 mutations was approximately 13%, birds on trial were annovulatory during the course of the trial (years 2-4 of life). In contrast, in the second trial, the birds ovulated throughout the course of their life, including the trial (years 2-4 of life). Thus, they had twice as many ovulations as the birds in the first trial. Given that we noted significantly greater p53 alterations in the second trial, the findings are thus consistent with what is known for human ovarian cancer, and is thus again supportive the chicken as a valid animal model for ovarian cancer.

Despite being among the most common targets of mutation in various types of cancer, codons 12 and 13 of H- and K-Ras are not mutated in chicken ovarian tumors. Similarly, human ovarian cancers rarely exhibit Ras mutations.

While more work needs to be done to characterize the chicken model for ovarian cancer, this work demonstrates the parallels of the molecular pathways in development of ovarian cancer in chickens and women and further supports the chicken model for ovarian cancer research.

B) Examination of HER-2/neu expression in chicken tumors
HER-2/neu staining has been completed on a subset of avian ovarian and oviductal adenocarcinomas. Sections of normal ovary and oviduct and adenocarcinomas of the ovary and oviduct of 4-yr old laying chickens in a reference set of reproductive tract tumors (Appendix 1) were stained for *Her 2/neu* and examined. A control adenocarcinoma of human origin was included. Three slides were prepared from each case, including positive and negative *Her 2/neu* stained slides and a histologic slide stained with hematoxylin and eosin. A subjective scoring system was created for individual tumors based on intensity and number of positive cells and for tissues based on the scores for the tumor nodules.

Tumor scores: 0 = no staining to minimal positive staining of less
           than 10% of the cells
        1 = mild to moderate positive staining of 10 – 50% of
         the cells
       2 = mild to moderate positive staining of >50% of

the cells

3 = moderate to marked positive staining of <50% of
the cells

4 = moderate to marked positive staining of >50% of
the cells

<u>Tissue scores:</u>   0 = no positive scores to <25% score 1 tumors

1 = >50% of tumor scores are score 1 with less than half score 2; not all tumors

positive

2 = >50% of tumors are score 2 with less than half score 3 or 4; most or all of
tumors positive

3 = >50% of tumors score 3 or 4; all tumors positive

Results

The pattern of *Her 2/neu* staining in chicken tissues was distinctly different from that of the control human adenocarcinoma. Staining of both human and chicken tumor cells was cytoplasmic; the nucleus was unstained. However, in the human tumor, staining of the cytoplasm was diffuse and the cell membrane was intensely stained. In the chicken there was multiple punctate staining in the cytoplasm, especially in the basal and apical parts of the cell when they lined tubules, and the cell membrane was not stained (Figures 1,2).

Significant positive staining was not restricted to tumor cells. The ovarian surface epithelium, adrenal cortical cells, neurons, and macrophages often stained intensely. A variety of other normal tissue cells, including liver and focal areas of oviductal mucosal epithelium, showed minimal to mild staining. Distinction between stained normal cells and tumor cells was easily accomplished based on location and morphology. Staining of the adrenal gland and sympathetic ganglion, which are embedded in to medulla of the left ovary had to be distinguished from tumor cells. Tissue and tumor cells that stained positive were not stained in the negative control sections indicating the staining for *Her 2/neu* was specific even though the pattern of staining was different (Figure 3).

Ten of 19 ovarian adenocarcinomas, but only 1 of 17 oviductal adenocarcinomas showed significant *Her 2/neu* staining (Table 1). Staining ranged from minimal to intense and varied substantially among the tumor nodules (Figures 4, 5). Small cells often stained more intensely than large cells, but this may have been relative due to concentration of stained structures in the smaller cells. Tumor emboli in thin-walled perifollicular vessels, interpreted as lymphatics, were usually intensely stained (Figure 6). An unexpected finding was the almost complete lack of staining of oviductal adenocarcinomas. More work would need to be done, but perhaps *Her 2/neu* could provide a means of distinguishing between oviductal and ovarian cancers, something that presently is not possible. All oviductal tumors with staining scores (1, 2, or 3) were advanced Type 3 tumors. Several of these had tumor emboli in perifollicular vessels. All of the Type 1 tumors did not stain along with the only Type 2 tumor in the series and four Type 3 tumors. This distribution suggests that *Her 2/neu* staining is more likely to be seen in advanced tumors. As expected, leiomyomas and leiomyosarcomas were not stained.

Table 1. Distribution of *Her 2/neu* staining scores for adenocarcinomas from laying hens.

	n =	Score 0	Score 1	Score 2	Score 3

Ovarian cancer, Type 1	4	4	*	*	*
Ovarian cancer, Type 2	1	1	*	*	*
Ovarian cancer, Type 3	14	4	3	4	3
Oviductal cancer	17	16	*	1	*

*No hens in this category

In summary, these findings indicate that *Her 2/neu* expression in ovarian cancer in the laying hen is comparable to that in affected women further substantiating the similarity of the chicken model to ovarian cancer in women. While the difference in staining pattern is somewhat disconcerting, the negative stain controls suggest the staining pattern is not an artifact. Differential staining of ovarian and oviductal tumors was unexpected and, if proven in more rigorous studies, could be a significant tool for distinguishing between these two common cancers in the chicken. Further studies to better understand *Her 2/neu* expression in the chicken are needed. Representative examples of staining are shown below.
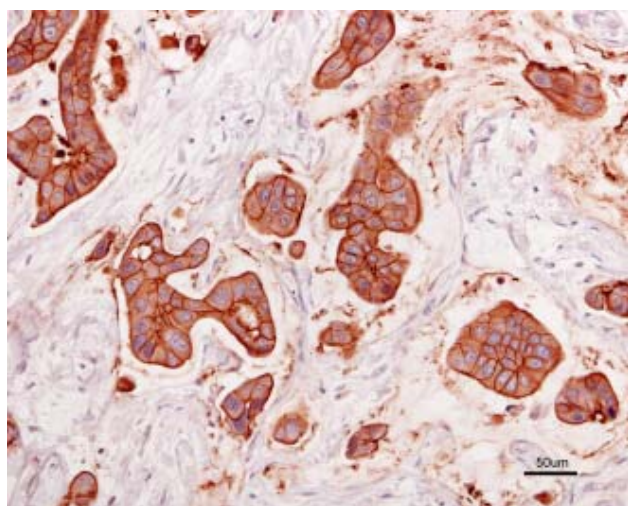

Figure 1. Adenocarcinoma, Human, *Her2/neu*, positive control. Diffuse cytoplasmic and intense cell membrane staining.
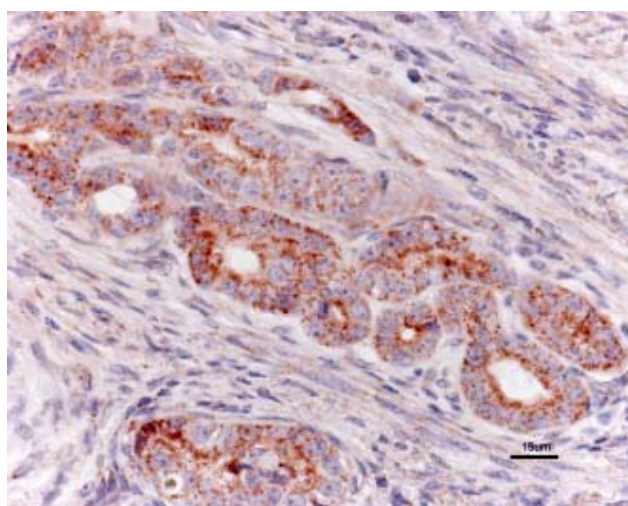

Figure 2. Adenocarcinoma, Ovary, Chicken, *Her2/neu*. Punctate staining in cytoplasm especially apical area of cells, nucleus and cell membrane not stained.
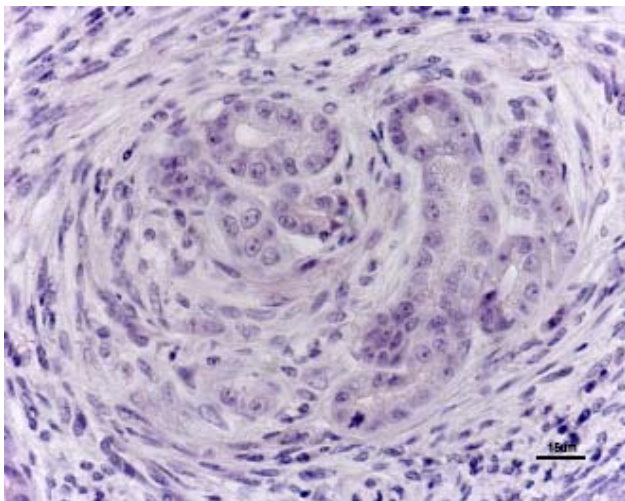
Figure 3. Same as figure 2, negative stain control. Lack of staining indicates positive staining is unlikely to be artifact.
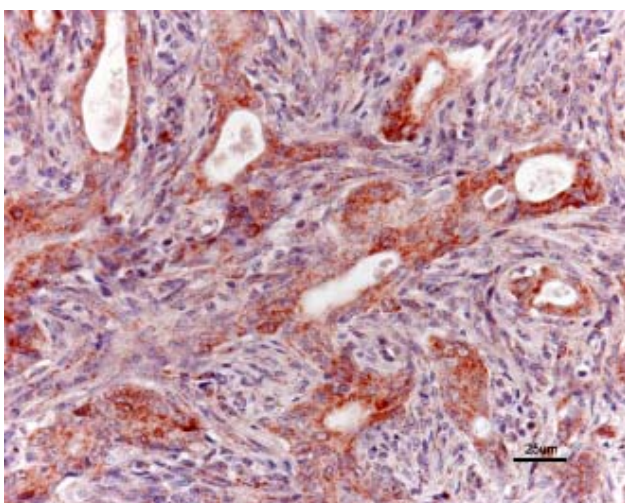

Figure 4. Adenocarcinoma, Ovary, Chicken, *Her2/neu.* Typical staining of tumor in an area of stromal hyperplasia.
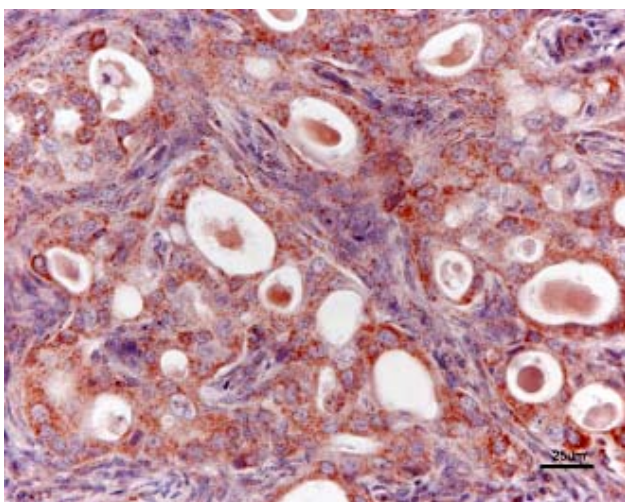

Figure 5. Adenocarcinoma, Ovary, Chicken, *Her2/neu.* Intense staining of tumor cells in a moderately differentiated (Grade 2) adenocarcinoma.
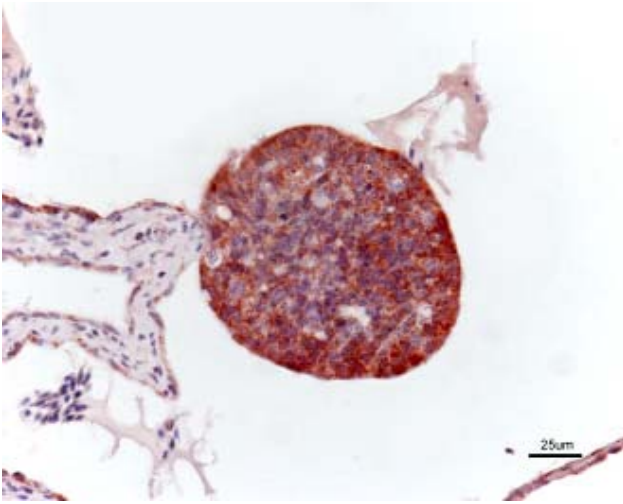
Figure 6. Adenocarcinoma, Ovary, Chicken, *Her2/neu.* Intense staining of cells in a tumor embolus in a perifollicular vessel consistent with a lymphatic.

Expression of TGF-beta isoforms in the chicken ovarian epithelium.

Transforming Growth Factor-beta is a molecule that has diverse biologic effects in vivo that are relevant to cancer prevention, including induction of apoptosis, differentiation, and inhibition of proliferation for many types of epithelia. Previously, we have demonstrated that progestin differentially regulates Transforming Growth Factor-beta in the ovarian epithelium. This discovery formed the basis for a hypothesis that a biologic effect of the progestin component of oral contraceptives (OC) may be responsible for the marked ovarian cancer protective effect of OC use. Specifically, we demonstrated in primates that progestins decrease expression of TGF-Beta in the ovarian epithelium, and that this is concomitant with an increase in expression of either the TGF-beta-2 or 3 isoforms. We have now examined expression of the three TGF-beta isoforms in normal ovaries from chickens on a prevention trial, receiving either no hormone (controls), versus the progestin levonorgestrel, versus the retinoid 4-HPR. Ovarian sections from the three treatment groups were stained with antibodies specific to the TGF-beta-1, 2, and 3 isoforms. Stained sections were then examined blindly under a multi-headed microscope by three investigators that were all blinded to treatment group data. The degree of intensity of staining for TGF-beta was then graded on a scale of 1+ to 3+ and recorded. Results demonstrated a significant decrease in expression of TGF-beta-1 in the ovarian epithelium in chickens receiving levonorgestrel relative to controls (p=0.0006). For TGF-beta-2, similarly, expression was decreased in association with both levonorgestrel and 4-HPR relative to controls, with additional significant decrease noted in levonorgestrel group relative to the 4-HPR group (p=0.0001). For TGF-B3, there is no difference in expression among the three groups (p = 0.3324). The data are shown below.

 The finding that progestins lower the incidence of ovarian cancer in chickens (from our first chemoprevention trial) and also induce changes in expression of TGF-beta that are similar to what we have observed in primates lends support to the chicken as a valid ovarian cancer animal model.

Statistical Analysis of TGF-beta Immunohistochemistry Study

| Antibody | Group | | | ANOVA |
	Control Mean(SD)	4HPR Mean(SD)	Levo Mean(SD)	
TGF-B1 [a]	n=29 2.0 (0.64)	n=29 1.53 (0.81)	n=28 1.20 (0.80) *	$F_{(2,83)}=8.18$ p=0.0006
TGF-B2 [b]	n=29 2.50 (0.63)	n=30 2.12 (0.59)	n=30 0.78 (0.68) *	$F_{(2,86)}=60.18$ p<.0001
TGF-B3	n=30 1.0 (0.69)	n=30 1.13 (0.68)	n=29 1.28 (0.75)	$F_{(2,86)}=1.12$ p=0.3324

[a]: the difference is between Control and Levo, with Tukey post hoc test.
[b]: the difference is between Control and Levo, between 4HPR and Levo, with Tukey post hoc test.

Aim 2: To classify the morphologic and histologic features of chicken ovarian cancers, leading to the development of a histologic classification of chicken ovarian adenocarcinomas.

Neoplasia of the female reproductive tract of chickens is common. Most frequently these are adenocarcinomas that spontaneously arise in the ovary, and/or oviduct (Fredrickson, 1987). A variety of tumors has been reported in the literature and seen in our studies on 7233 hens during the past 5 years. No single scheme for classifying these tumors has been devised. The objective of this study has been to develop a classification scheme for neoplasms that affect the avian female reproductive tract (see Table 1). In this report emphasis is given to malignant epithelial tumors because of their potential to serve as a model of ovarian cancer in woman. The report will conclude with a summary of the model.

Materials and Methods

Source of tumors. Reproductive tract tumors were collected from 7233 laying hens used in three ovarian cancer trials. Trial 1 was a cross-sectional study of 1405 4-yr-old hens at the conclusion of a chemoprevention trial (154 tumors, 11%). Trial 2 consisted of a longitudinal study of 980 normal hens between 8 and 28 months of age (40 tumors, 4.1%) and a cross-sectional study of 2002 normal 28-month-old hens that had experienced different molting methods (185 tumors, 9.2%). Trial 3, a second chemoprevention trial, also had two parts - a longitudinal study of 2170 hens between 18 and 48 months of age (857 tumors, 39.5%) and a cross-sectional study at the conclusion of the study of 676 hens at 4 years of age (276 tumors, 40.8%).

Pathology. Hens that died during the longitudinal studies were removed from their pens as soon as possible after death. They were identified by date and treatment using waterproof tags (Tyvek®, Uline, Waukegan, IL 60085) and placed into an ice water bath for 1-2 hrs. The goal was to reduce the core temperature to <10° C to decrease decomposition to a negligible rate. After chilling, carcasses were placed into individual plastic bags and refrigerated. Birds that had died were necropsied weekly to determine the most probable cause of death and examine the hens for tumors. Samples of tumors from well-preserved carcasses were taken and fixed in 10% buffered neutral formalin.

Hens in cross-sectional studies were euthanized by cervical dislocation and necropsied. The oviduct was straightened by separating it from the dorsal and ventral mesosalpinx, opened along its length, and the mucosa examined for tumors. The ovary was examined, removed, and carefully examined for any abnormal masses. Other organs were examined for lesions, especially if tumors had been

found in the reproductive tract. Samples of   tumors from reproductive tracts and other tissues of hens that were euthanized were collected, fixed for 72-hours in 10% buffered neutral formalin, and transferred to 70% alcohol. The ovary was re-examined for small tumors when it was trimmed for histopathology. Ovary and oviduct samples were collected and processed even if no tumors were grossly evident from approximately 10% of the birds in each group. The ovary and oviduct were examined histologically from all birds (n = 425, 86 tumors, 20.2%) in two groups in the first study regardless if gross lesions were identified or not. Additional samples of tumors and normal tissues from selected birds were snap frozen at the time of necropsy for genomic analysis.

Slides were prepared and stained with hematoxylin and eosin following conventional paraffin embedding and sectioning for characterization. Selected tissues also were stained with periodic-acid Schiff, Giemsa, Gomori's methenamine silver, reticulin, Alcian blue, toluidine blue, phosphotungstic acid, Verhoff-van Gieson's, and Masson's trichrome stains. For immunostaining 40 cases representing normal tissue, ovarian cancer only, oviductal cancer only, and ovarian and oviductal cancers were identified (Appendix 1). Sections for immunohistochemistry were mounted on Super Frost + ® slides (Fisher Scientific, Raleigh, NC) and stained with antibodies for pan-cytokeratin (AE1/AE3), cytokeratin 7, cytokeratin 20, vimentin, smooth muscle actin, ovalbumin, estrogen, progesterone, and FSH receptors, CA 125, and WT-1. Antigen retrieval and use of antibodies were done according to manufacturer's instructions.

Results and Discussion

Clinical signs. Monthly mortality remained fairly constant during the longitudinal studies, but the percent of hens with cancer among those that died increased rapidly after 2 years of age (Figure 7). The youngest hen found to have a cancer was 14 months.
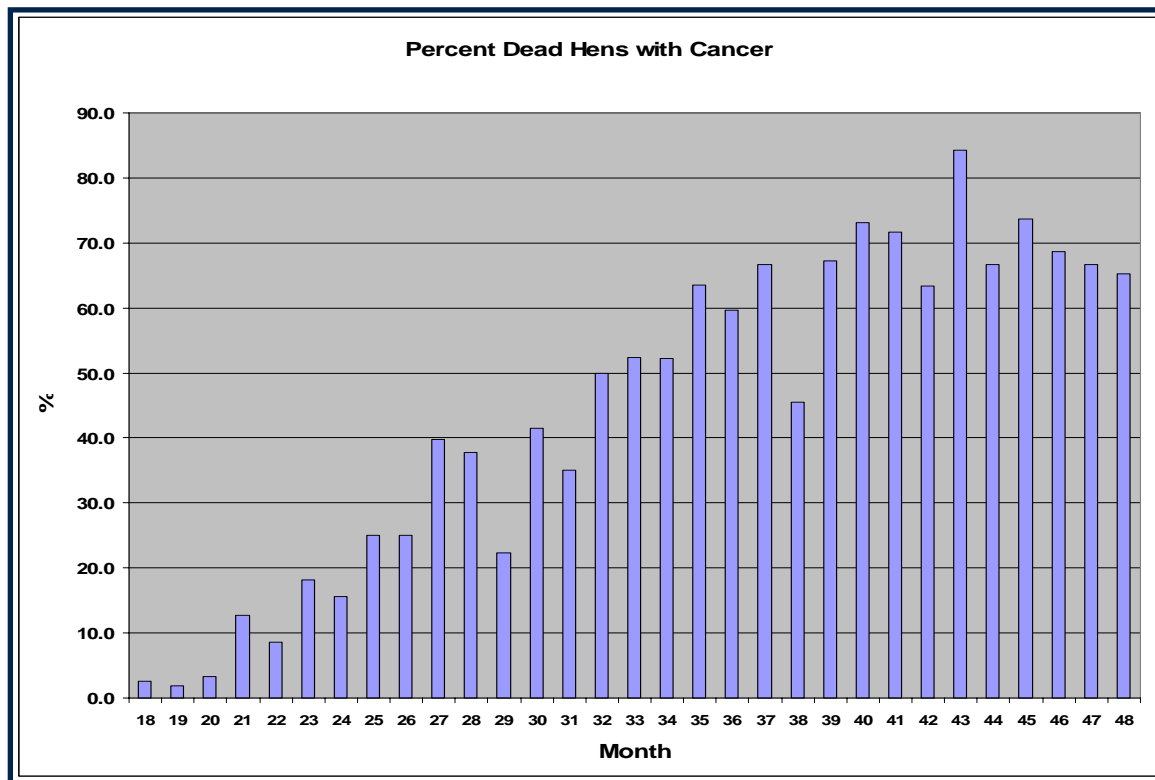

Figure 7. Trial 3, longitudinal study, 2170 hens that died between18 and 48 months of age.   Monthly mortality did not vary greatly (data not shown) but the number of birds that had cancer among those

that died increased progressively with age.   Between 32 and 48 months of age, cancer accounted for more deaths than all other causes combined except for month 38.

Unless the tumor was advanced or there was concurrent peritonitis, affected hens could not be distinguished from unaffected hens. They generally were in good condition and continued to ovulate from unaffected areas of the ovary (Figure 8).

Figure 8. Hens with ovarian cancer often remain clinically normal and continue to ovulate from unaffected areas of the ovary. Above is shown three follicles at different stages of development, with the dominant follicle that is destined to ovulate leading to the next egg that is layed shown in lateral right portion of photo.

Eggs were produced if the oviduct had not become obstructed by an oviductal tumor. Hens with advanced reproductive tract tumors often developed extensive ascites, which caused the birds to have an upright "penguin-like" stance (Figure 9). Muscle atrophy was a common finding in advanced cases. Even though the bird was thin, its weight was often above average because of concurrent ascites.

Figure 9. Ascites commonly develops in hens with advanced cancer. The additional weight causes the hen to assume an upright 'penguin-like' stance.

Gross pathology. Tumors that did not involve the reproductive tract were rare in hens with cancer. In one series 96% (413/430) of affected hens had lesions in either the ovary and/or oviduct. In a second series of hens that died between 2 and 4 years of age, 98.6% (272/276) of the hens with cancer had tumors of the reproductive tract. Adenocarcinomas occurred simultaneously in the ovary

and oviduct more frequently than in either organ   alone. Among the 413 reproductive cancers in the study noted above, 67 (16.2%) were only in the ovary, 99 (24.0%) were only in the oviduct, and 247 (59.8%) were in both the ovary and oviduct. Collectively, tumors of the oviduct occurred slightly more frequently than tumors of the ovary (76.0% and 83.8% respectively). Hens with reproductive tract adenocarcinomas often had moderate to marked ascites and generalized serosal tumors involving most frequently the intestinal peritoneal cavity (carcinomatosis) (Figure 10).

Figure 10. Generalized tumors involving the intestinal peritoneal cavity (carcinomatosis) is often seen in advanced cases. The lesion is analogous to "omental caking" in women as birds do not have an omentum.

Extensive lesions resulted in local invasion of the duodenum and pancreas causing fibrosis and distortion of tissues (Figure 11).

Figure 11. Invasion of the duodenum and pancreas is common because of their location in the intestinal peritoneal cavity ventral to the ovary and oviduct.

An infrequent complication was intestinal obstruction. In addition to the peritoneum, metastasis to the liver and lung occurred in approximately 20-30% of hens with reproductive tract cancer (Figures 12,13).
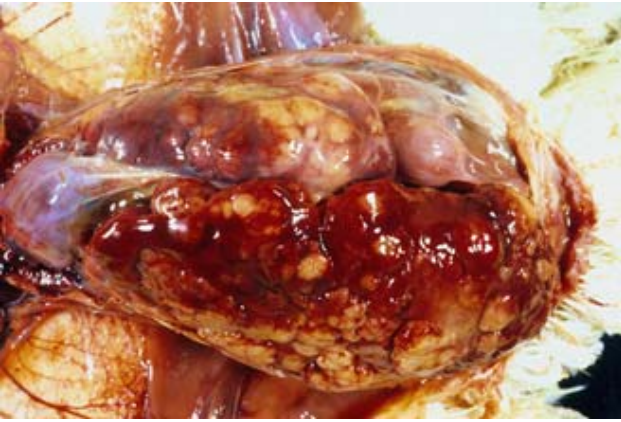
Figure 12. Metastasis to the liver occurs in approximately 20% of hens with reproductive tract cancer.

Figure 13. In addition to the liver, approximately 20-30% of hens with reproductive tract cancer have metastasis to the lungs.

Adenocarcinomas in the ovary were firm to hard, multilobular, irregular, solid or pedunculated, and light tan to cream colored (Figures 14,15). They frequently had a "cauliflower-like" appearance. Some nodules were umbilicated. Cysts containing clear, yellow, red, or green fluid were often present on the surface.

Figure 14. Typical ovarian cancer prior to generalized spread in the body cavity or to other organs. Hen has normal fat deposits and is in production.

Figure 15. Ovarian cancers with follicular regression from two hens. Tumors are multilobular, irregular, solid or pedunculated, and light tan to cream color.

Approximately 5% of ovarian tumors presented as cystadenocarcinomas with either numerous large or small cysts throughout the tumor (Figures 16,17). Other tumors that occurred infrequently or rarely in the ovary were granulosa cell tumors, sex cord tumors, and teratomas.

Figure 16. Cystadenocarcinoma – large cyst form. Multiple cysts comprising the tumor fill the body cavity of this hen. Approximately 5% of ovarian cancers are cystadenocarcinomas

Figure 17. Cystadenocarcinoma – small cyst form. Multiple small cysts are present throughout the tumor.

Adenomas and adenocarcinomas of the oviduct   that arose from the glandular epithelium in the magnum were the most common epithelial tumors of the reproductive tract. It was not always possible to differentiate adenomas from adenocarcinomas on gross examination, but there were several characteristics of each one that helped in differentiating them. Adenomas were solitary or multiple and typically had a smooth surface, were round to oval, generally <1 cm, and uniform color. Often adenomas presented as narrow-based pedunculated polyps, but sessile, broad-based lesions also occurred (Figure 18).

Figure 18. Adenomatous polyp in the oviduct. Transition from adenomas to adenocarcinomas indicates they are pre-neoplastic.

Occasionally the oviduct mucosa had large numbers of adenomatous polyps consistent with adenomatosis (Figure 19).
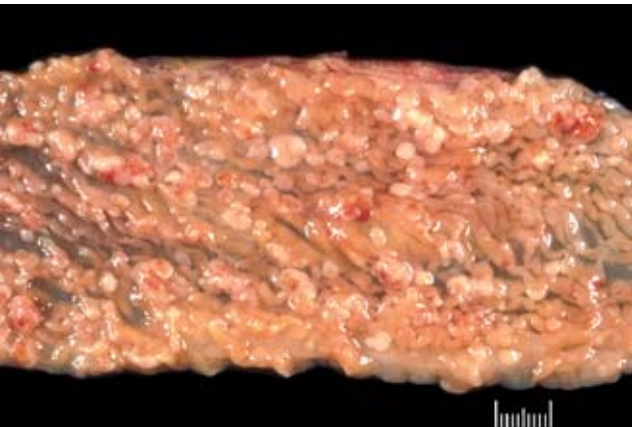

Figure 19. Adenomatosis of the oviduct. Numerous polyps characterize this condition. Larger polyps are developing features of adenocarcinomas.

In contrast, oviductal adenocarcinomas were   irregular, generally >1 cm, had areas of different colors, and were sessile to intramural; rarely occurring as polyps. Typically they appeared as thickened, firm areas with irregular lobular or nodular patterns in the oviduct wall (Figures 20,21). Occasionally cancers extended either extra- or intraluminally but, on microscopic examination, mural oviductal tumors did not breach the mucosal epithelium. A rare epithelial tumor, distinct from adenomas and adenocarcinomas that developed from mucosal rather than glandular epithelium was a cystadenoma of the oviduct infundibulum.
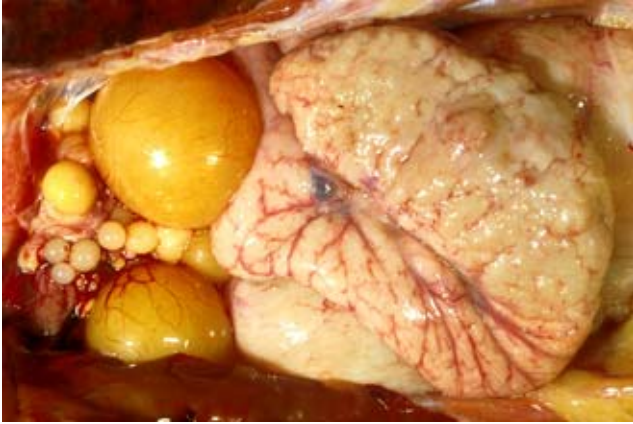

Figure 20. Large oviductal adenocarcinoma, normal ovary. Size of tumor and absence of tumors in other tissues indicates rapid growth.

Figure 21. Oviductal adenocarcinomas. Tumors develop intramurally, invade, and expand into the lumen or body cavity

The only other reproductive tract tumors that were found frequently were leiomyomas. These developed from smooth muscle in the muscle layer of the oviduct, tunica media of large arteries, free margin of the ventral mesosalpinx, or the mesovarium/ovarian medulla. Leiomyomas that arose from the oviduct muscle layer were often multiple, round to oval, smooth, firm, translucent, pale color, and <2cm (Figure 22).

Figure 22. Multiple leiomyomas arising from muscle layer of the oviduct. Smooth, glistening, translucent appearance are characteristic.

Those in the mesosalpinx were single, larger, and more irregular. Some had numerous varicose veins radiating from the tumor (Figure 23). Another mesenchymal tumor type identified in the oviduct was fibroma, which was rare.
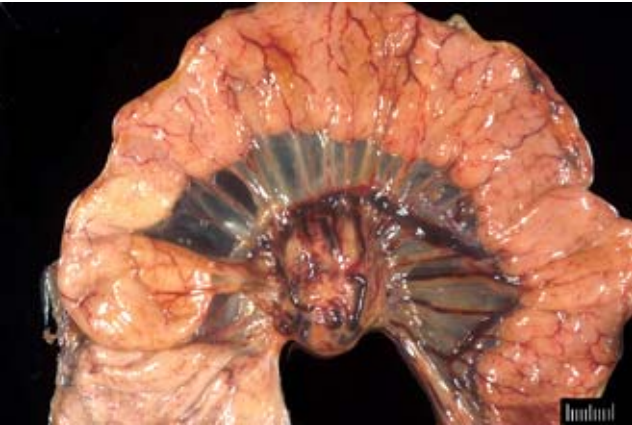

Figure 23. Leiomyoma of the free margin of the ventral mesosalpinx with large varices radiating from the tumor. Not all leiomyomas have varices.

Histopathology. *Adenocarcinoma.* Microscopically, adenocarcinomas in the ovary, oviduct, or both reproductive tissues were similar. The basic pattern was lobules or nodules composed of short tubules or spherules lined with a secretory, simple, cuboidal, or low columnar epithelium that typically contained eosinophilic proteinaceous fluid in the lumen (Figure 24). They had variable amounts of interstitial fibrovascular tissue and/or smooth muscle bundles, which accounted for their scirrhous nature grossly.
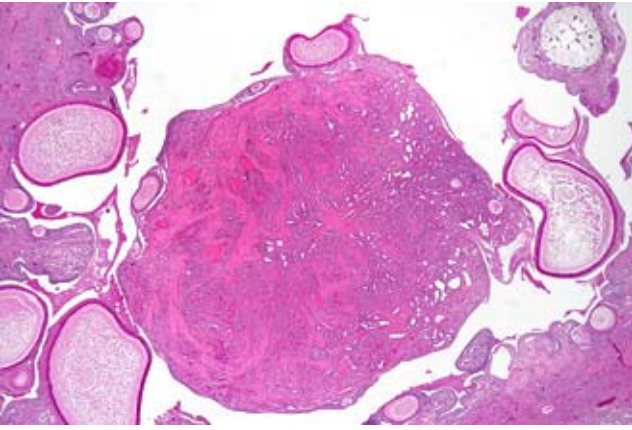
Figure 24. Ovary, Adenocarcinoma. Cortical tumor nodule arranged in lobules composed of short tubules or spherules separated by an irregular network of connective tissue and smooth muscle. Proteinaceous fluid fills the lumen of some tubules.

The amount of interstitial fibromuscular tissue did not correlate with the degree of tumor differentiation and could vary considerably among multiple tumors in the same bird (Figure 25).
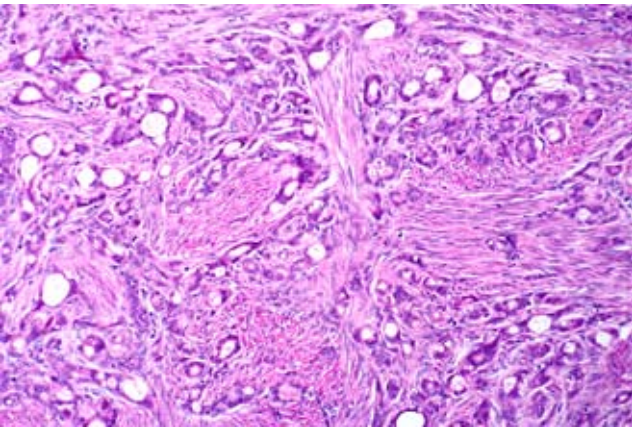

Figure 25. Peritoneum, Adenocarcinoma. Interstitial fibrovascular tissue and/or smooth muscle bundles account for the scirrhous nature grossly and do not correlate with the degree of tumor differentiation

Squamous differentiation, osseous metaplasia, and cystic and/or papillary patterns occurred infrequently. Often tumors could be observed grossly on the surface of ova, but microscopically they were in perifollicular spaces, considered to be lymphatics, and did not penetrate the follicle wall (Figure 26). Mitosis was uncommon to rare except in more anaplastic areas where cells were found in sheets rather than tubules.
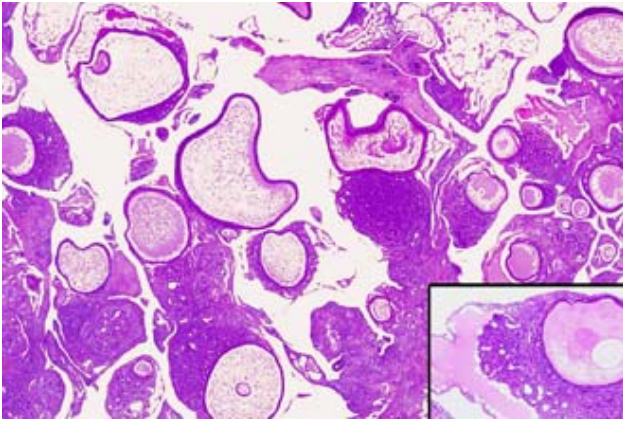
Figure 26. Ovary, Adenocarcinoma. Ova are spared. Tumor cells are in perifollicular spaces interpreted to be lymphatics. Inset shows tumor adjacent to an ovum within a dilated space containing protein-rich fluid.

To account for the simultaneous presence of well-developed tumors in the oviduct and ovary in the same hen and the fact that primary ovarian tumors have markers previously considered specific for oviductal adenocarcinomas, we developed a typing system for ovarian adenocarcinomas. Type I ovarian adenocarcinomas were restricted to the cortical surface as small perifollicular, miliary nodules that comprised <25% of the ovarian tissue. These were considered metastatic lesions to the ovary if tumors were present in the oviduct. If no tumors were present in the oviduct, they were interpreted as early primary ovarian cancers. Type II ovarian adenocarcinomas involved both the cortex and medulla and comprised >25%<75% of the ovary (Figure 27).
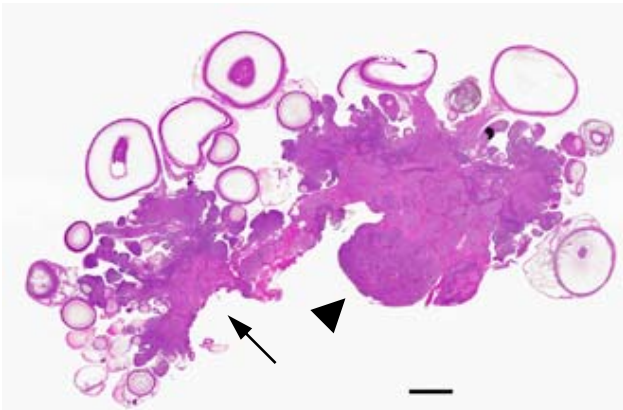

Figure 27. Ovary, Adenocarcinoma, Type 2. Miliary cortical nodules typical of secondary tumors (arrow) and larger tumors in the cortex and medulla typical of primary tumors (arrowhead).   Bar = 2 mm

They were interpreted as probable primary tumors. Type III ovarian adenocarcinomas comprised >75% of the ovary and were interpreted as primary adenocarcinomas. Type II and Type III adenocarcinomas were classified as such independent of the presence or absence of adenocarcinomas in the oviduct. Tumors of the ovary often occurred in conjunction with oviductal tumors suggesting a possible synchronous (multicentric) origin rather than spread by metastasis from a primary tumor to other sites and tissues. Multicentric tumors of other types are well documented in chickens.

A scheme to classify adenocarcinomas according to their degree of differentiation was developed based on architectural pattern, degree of cell differentiation (production of ovalbumin granules and secretion of proteinaceous fluid), and appearance of the epithelial cells. Using this scheme it was possible to classify adenocarcinomas of the reproductive tract into three grades. Grade 1 tumors were well-differentiated tumors characterized by a well-developed architectural pattern of tubules or spherules that were lined by reasonably uniform cuboidal to low columnar epithelial cells. Mitotic

figures were absent to rare. If the hen was in   active production, the cytoplasm of the cells typically contained variable numbers of ovalbumin granules. However, the number of granules in the cytoplasm of tumor cells was less than that in the cytoplasm of normal oviductal glandular epithelium (Figure 28).
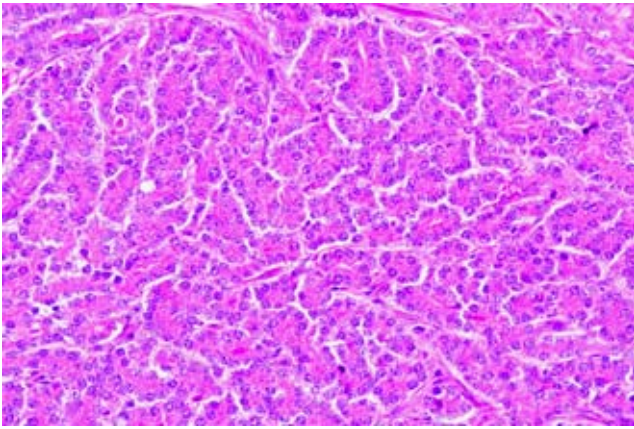

Figure 28. Ovary, Adenocarcinoma, Grade 1. Well-differentiated tumor composed or tubules lined by uniform epithelial cells that have basal nuclei and abundant cytoplasmic ovalbumin granules.

Grade 2 tumors were differentiated but not as well as Grade 1 tumors. These tumors were characterized by recognizable tubules and spherules, but they were often irregular or poorly formed and were lined by cells that lacked uniformity, often showing crowding or piling along the basement membrane. Mitotic figures were rare to occasional. Cytoplasmic granules were often limited to focal areas and secretory material was minimal to absent in some tubules (Figure 29).
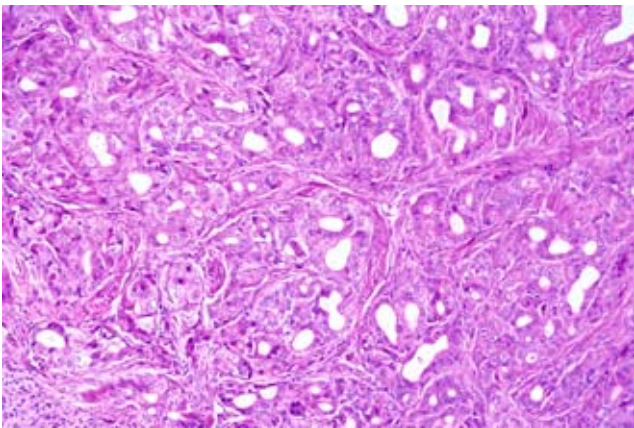
Figure 29. Ovarian Adenocarcinoma, Grade 2. Tubular pattern is apparent but tubules are irregular and epithelial cells are not uniform. Lack of granules and albuminous fluid in tubules indicate hen was not in production.

Grade 3 tumors were poorly differentiated often containing highly pleomorphic cells devoid of cytoplasmic granules in sheets or very poorly formed tubules and spherules. Mitotic figures were often numerous (Figure 30). It was common for different tumors within the same bird to exhibit differing degrees of differentiation, but generally only within two grades. Occasionally differences in differentiation spanning two grades could be identified within lobules of a single tumor. There were no consistent differences in Grade between presumed primary and secondary tumors.

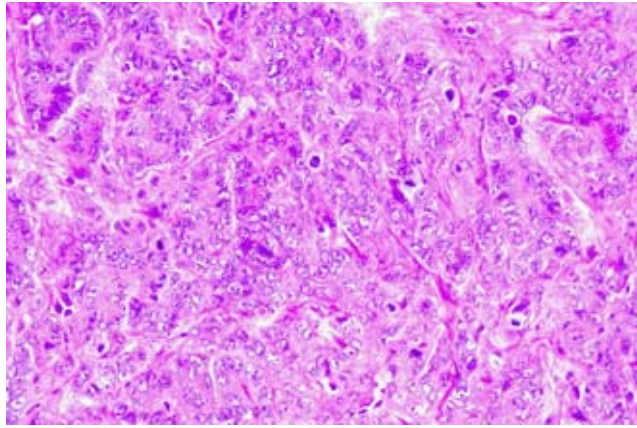Figure 30. Peritoneum, Ovarian Adenocarcinoma, Grade 3. Tubular pattern is barely recognizable. Cells are highly variable and there are numerous mitotic figures.

Except for poorly differentiated anaplastic Grade 3 adenocarcinomas, it was generally possible to find at least a few cells that had cytoplasmic ovalbumin granules characteristic of those in oviductal glands if the hens were in production. When egg laying ceased, the number of ovalbumin granules in both normal oviductal glandular epithelium and tumor cells decreased to the point that they were no longer detectable in tumor cells. The parallel changes in the number of ovalbumin granules between normal and tumor cells related to production status suggests that neoplastic cells were responding to the same hormonal signals as normal glandular epithelial cells. Ovalbumin granules showed polychromatic staining with Masson's trichrome stain. Immature granules were deeply basophilic while mature granules were eosinophilic (Figure 31). How the tumors develop and grow on serosal surfaces could be determined by examining the staining character of the cytoplasmic granules.
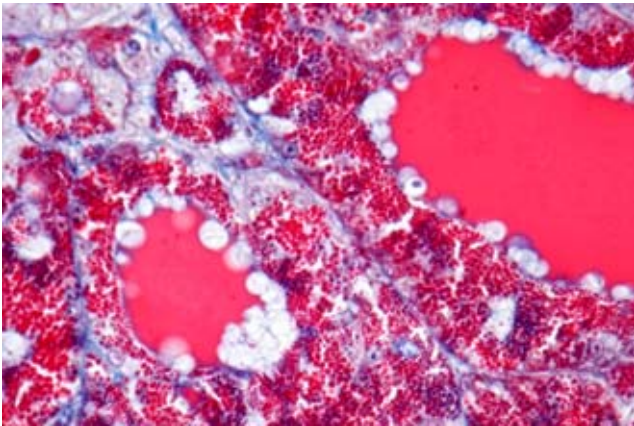
Figure 31. Ovary, Adenocarcinoma, Grade 1. Ovalbumin granules within the cytoplasm of tumor cells stain brightly with Trichrome stain. Immature granules are basophilic.

Presence of ovalbumin granules in the cytoplasm of ovarian tumor cells previously was considered a specific marker that the ovarian tumor was not primary and that the primary tumor was in the oviduct (Haritani *et al.* 1984). Similarly, absence of ovalbumin granules was interpreted to indicate a tumor in the ovary was primary. Our studies showed that presence or absence of ovalbumin granules in adenocarcinomas of the ovary does not define where the tumor originated. A number of ovarian adenocarcinomas were obtained from hens in which careful gross and microscopic examination of

the oviduct failed to reveal any evidence of   neoplasia, but the characteristics of the tumor cells in the ovary were those of an oviductal glandular epithelium type. Our findings indicate these are true ovarian tumors composed of oviductal-like cells. These findings recently have been independently corroborated (Giles *et al.*, 2004*)*.

Our findings and those of Giles *et al.* indicate ovarian adenocarcinomas in chickens differentiate in a similar way to ovarian adenocarcinomas in women. Adenocarcinomas of women can be composed of cells with characteristics of oviductal (serous), uterine (endometrioid), cervical (mucous), or bladder (transitional) epithelium. Similar to ovarian cancers in women, those in chickens also undergo Müllerian duct differentiation. Dissimilarity in cell morphology between chicken and human ovarian tumors can be attributed to anatomical and functional differences. The mammalian reproductive tract provides an environment for supporting fetal development whereas the avian reproductive tract is primarily secretory.

Serosal implantation was evident in most hens that had peritoneal tumors. The earliest identifiable change was a cluster of cells on or just beneath the serosal surface. Tumor cells were large, oval, and had a pale finely granular cytoplasm. As these nests of tumor cells increased in size and cellularity, they spread into the deeper layers of the serosa and quickly initiated tubule and spherule formation. Rarely, necrosis of cells within the nests was seen. Continued development resulted in an expanding nodule within the serosa and appearance of cytoplasmic ovalbumin granules in the cytoplasm of the tumor cells if the hen was in production. At this stage, the granules were deeply basophilic when stained with Masson's trichrome stain. These cells became centrally located as additional new cells proliferated around the periphery and the granules shifted from basophilic to acidophilic. In almost all instances tumor implants developed in sites of previous inflammation. It is difficult to interpret this observation as mild peritonitis due to yolk entering the body cavity during follicular atresia is common in laying hens, but it may be possible that tumor cells require factors associated with inflammation for their survival and multiplication.

Cells that give rise to ovarian adenocarcinomas in chickens are currently unknown. There is a clear progression of histologic alterations in oviductal glandular epithelium from normal to neoplastic (focal or multifocal hyperplasia → dysplasia → adenoma [often polypoid] → adenocarcinoma). Such a progression is not evident in ovarian adenocarcinomas. It is prudent to assume that the source of ovarian tumors in the chicken is also the specialized ovarian surface epithelium that is considered to be the source of ovarian carcinomas in women until proven otherwise. However, an alternative hypothesis based on our observations in the chickens for the development of ovarian adenocarcinomas is possible. In this hypothesis free epithelial cells ascend the oviduct by retrograde propulsion and are expelled into the body cavity in proximity to the ovary. In hens the cells are glandular epithelial cells whereas in women they are mucosal epithelial cells from various locations within the genitourinary tract. In hens they become trapped in the many folds and convolutions that characterize the avian ovary; in mammals they are trapped within the ovarian bursa. Free epithelial cells could actually be deposited on the follicle at the time it bursts as the follicle is covered by the infundibulum in the hen. Normally these cells would die. However, if there was a site of inflammation, such as the physiologic inflammation that occurs during ovulation or mild yolk peritonitis, the cells might adhere to the fibrin or other substances, be nutured by inflammatory secretions, and survive. Cytokines produced locally as part of the inflammatory process could promote their multiplication. As the epithelial-mesenchymal trophic unit in their resident location would no longer control the cells, they could undergo transformation and ultimately develop into a neoplasm. The sequence of events that we believe may occur in the proposed Inflammation Implantation Tumorigenesis hypothesis is shown in Figure 32.
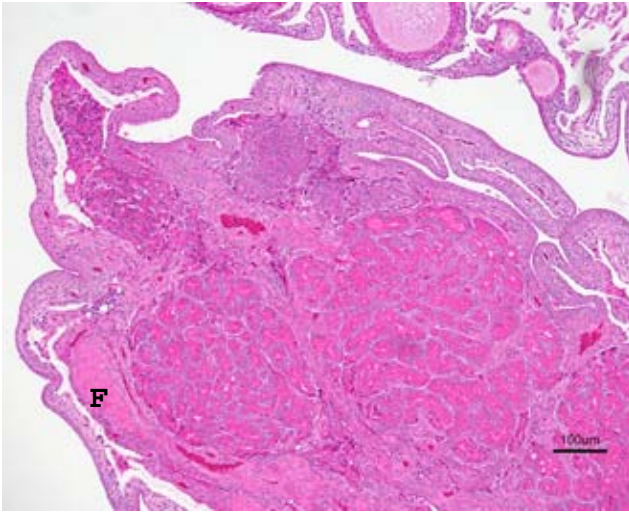
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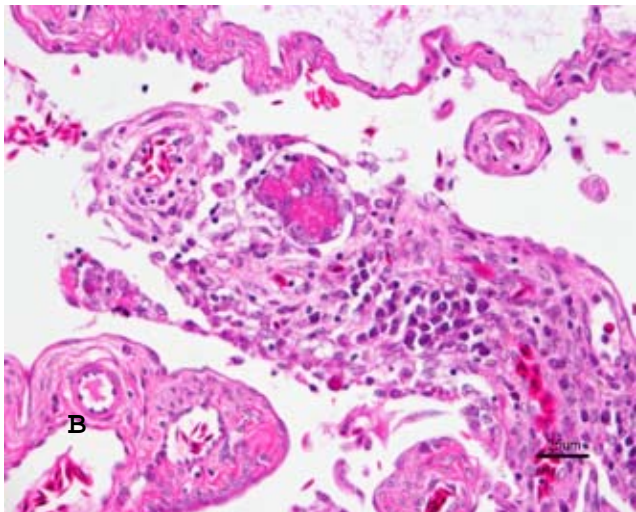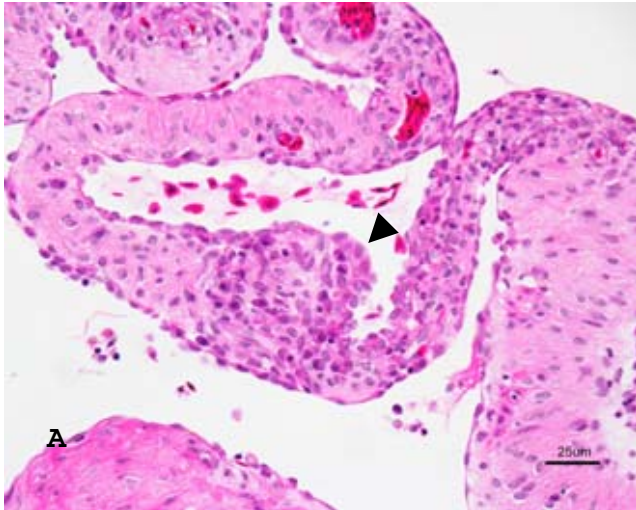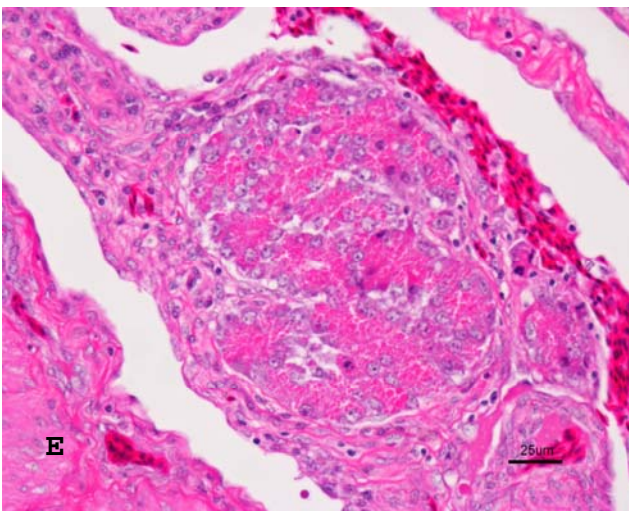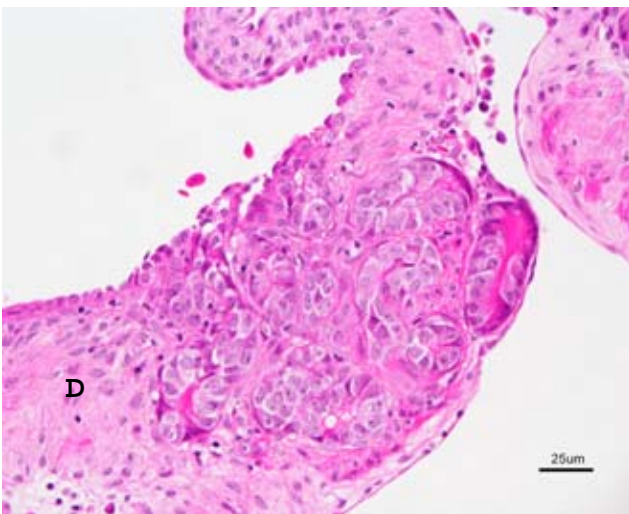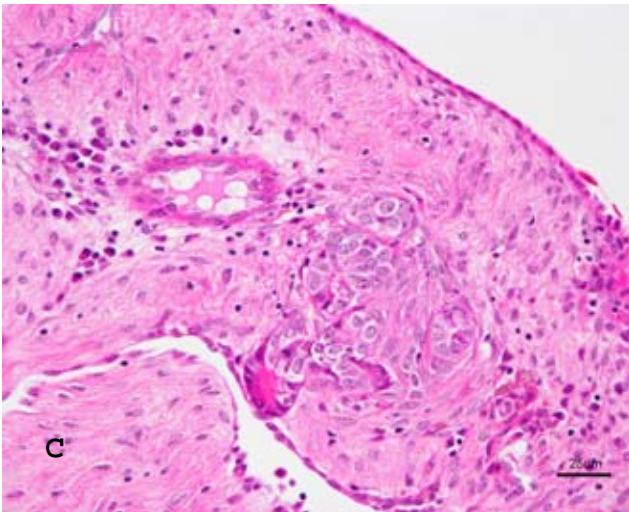
Figure 32(above). Alternative Ovary, Inflammation Implantation Tumorigenesis Hypothesis. A. Thickened focus in an area of inflammation with large epithelial cells on the surface (arrowhead). Without specific markers it is presently not possible to identify the specific cell type. B. Follicle typical of oviduct glandular epithelium in a focus of inflammation without large epithelial cells. C,D. Follicles of normal appearing oviductal glandular epithelium with large epithelial cells typical of those in early carcinomas extending from them. E. Small expanding well-differentiated adenocarcinoma typical of a Grade 1 lesion in same tissue location as lesions in C and D. F. Adenocarcinomas enlarge and extend into adjacent vascular spaces leading to disseminated ovarian cancer.

It has been accepted that spread of reproductive tract adenocarcinomas is either exclusively or primarily by implantation of serosal surfaces and, when metastatic lesions occur in organs, they develop from invasion of serosal implants (Fredrickson, 1987). Our studies indicate that while serosal implantation does occur, significant dissemination of the tumors occurs via the vascular system, most likely lymphatics. Tumors developed within the peritoneum or organ vessels and had no contact with the serosal surface. Progression of tumor development within vessels was similar to that of serosal implants, the primary difference being the location of lesion development (Figures 33,34). A surrounding vessel could only be identified in early lesions as it became obscured as the tumor expanded. Definitive evidence of vascular spread was found in the lungs where spread by implantation was not a possibility (Alfonso *et al.*, 2005). Based on occurrence of tumors in multiple organs in advanced cases, it is probable that vascular dissemination of tumors occurs late. Thrombosis of lymphatics by tumors may be responsible for the massive ascites that commonly occurs in advanced cases rather than fluid secretion by the tumor cells as has been thought previously.
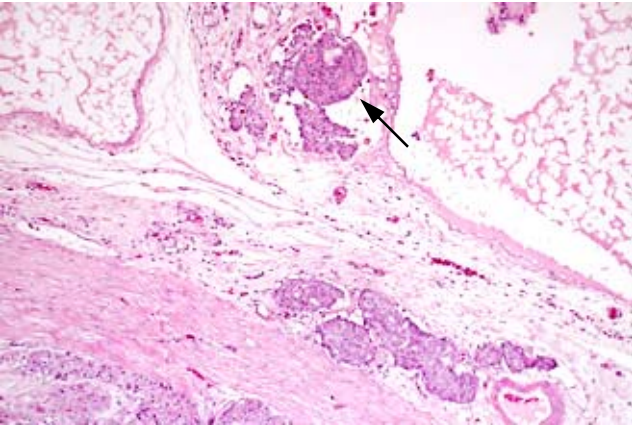
Figure 33. Adenocarcinoma emboli in small vessels in the mesentery and serosa of the intestine. Ascitic fluid in the body cavity. Tumor cells in the mesenteric vessel are forming tubules and secreting protein-rich fluid (arrow).
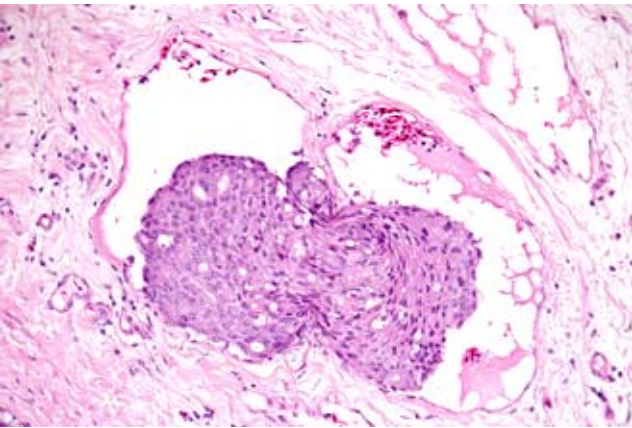
Figure 34. Same case as 33. A tumor embolus has lodged in a valve within a vessel and is expanding. Eventually the vessel will become filled with the tumor to the point that it will no longer be recognizable.

In addition to tumor spread by implantation and lymphatics, spread within the autonomic nerves in the intestines was seen when there had been extensive peritoneal tumor development and downward invasion of the pancreas and duodenum.

Adenoma. Benign epithelial adenomas were commonly found in the oviduct but were not identified in the ovary. Evidence of malignancy to definitively differentiate adenocarcinomas from adenomas relied on identifying invasion of adjacent tissues (lamina propria, submucosa, muscle layers, or vessels). For small intramural lesions, this depended on the section containing an area of tissue invasion. In polypoid lesions where tissue invasion was less likely, the criteria used were overall tissue organization, uniformity of cells, and cell appearance. Rarely adenomas contained distinct foci of carcinomatous tissue. Based on the findings in this study, adenomas should be considered pre-cancerous lesions.

Distinguishing hyperplastic glandular foci from   mural adenomas, especially when they contained nests of dysplastic cells was often difficult. Adenomas tended to compress adjacent glandular tissue, have an arrangement of glands that was more haphazard than the orderly arrangement of those in a hyperplastic focus, and were generally circumscribed although not encapsulated. Early adenocarcinomas and adenomas typically had little interstitial mesenchymal tissue. However, if interstitial connective and smooth muscle tissue was prominent, this was interpreted to be a feature of an adenocarcinoma. In common with adenocarcinomas, adenomas occasionally developed cystic and/or papillary architectures.

Other tumors. Granulosa cell tumors were characterized microscopically by their fine fibrovascular stroma, organization into nests or follicles having either no lumen or a lumen surrounded by multiple cell layers, and large round to oval cells that typically had a distinct eosinophilic margin and central nucleus. Structures resembling poorly formed Call-Exner bodies were rarely seen in a few tumors and were not useful in identifying a tumor as being of granulosa cell origin. Ovarian surface epithelium was often hyperplastic when a granulosa cell tumor was present.
Other sex cord tumors could be divided into Sertoli cell, seminiferous, and mixed types (Figure 35). They generally were small, oval to round, and solitary to multiple. Typically they occurred only in the cortex. In rare instances spermatozoa were seen in seminiferous sex cord tumors. These tumors were distinct from dysgerminomas in women as they had distinct tubular organization, lacked uniformity, did not occur in sheets, and rarely produced spermatozoa. Seminiferous sex-cord tumors had to be distinguished from ovotestes. Ovotestes had well-developed testicular tissue, usually producing spermatozoa, and immature ovarian tissue, whereas seminiferous sex cord tumors were minor components of otherwise well-developed ovarian tissue.
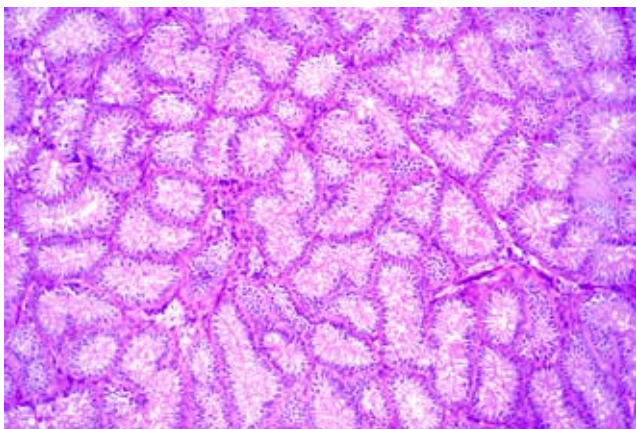
Figure 35. Sex cord tumor, Sertoli cell type. Well-defined tubules are uniformly lined by basally oriented cells that have a wispy, indistinct luminal border.

Leiomyomas were composed of well-differentiated interlacing bundles of hypertrophied smooth muscle cells that arose from normal smooth muscle in the tissues (Figure 36). A small percentage of tumors with the characteristics of leiomyomas contained small amounts (<10%) of neoplastic epithelial cells. It is unclear if these represent a distinct tumor type or the extreme spectrum of a scirrhous adenocarcinoma. In order to track these unusual tumors they were named 'leiomyocarcinoma' so they would not be included with more typical adenocarcinomas. Histologically, they are similar to fibroadenomas in women, but whether they are true counterparts remains to be determined.
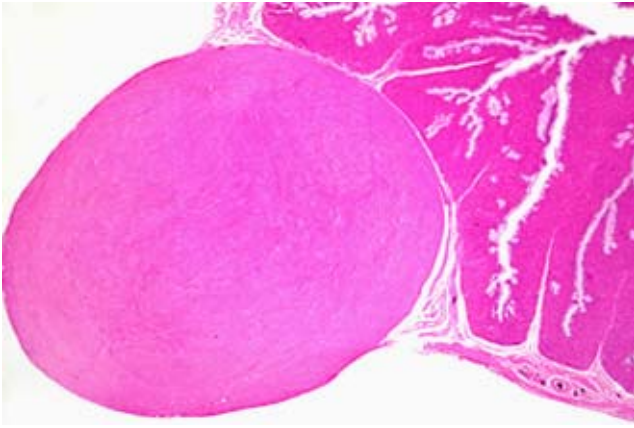
Figure 36. Leiomyoma arising from the smooth muscle in the muscle layer of the oviduct wall. Similar lesions occasionally contained neoplastic epithelium and were named leiomyocarcinomas.

Histochemistry and Immunohistochemistry. Of the histochemical stains only Alcian blue and Masson's trichrome provided additional information beyond that obtained by conventional hematoxylin and eosin staining. Alcian blue selectively stained the oviduct epithelium, which was useful in distinguishing paraoviductal cysts, which did not stain, from infundibular cystadenomas, which stained positive. Also, it further confirmed that the tumors in the ovary and oviduct that had characteristics of glandular epithelium were not of mucosal epithelial origin. As noted above Masson's trichrome was useful in identifying ovalbumin granules and determining their maturity.

Cells in both ovarian and oviductal adenocarcinomas stained positive for pan-cytokeratin (AE1/AE3), confirming their epithelial nature (Figure 37). Cells with cytoplasmic granules and secretory product within tumors stained positively using an ovalbumin antibody (Sigma-Aldrich, St. Louis, MO) (Figure 38). As discussed above, ovalbumin should not be considered specific for oviductal adenocarcinomas because the amount of ovalbumin in cells is related to reproductive state and is not detectable in tumors if the hen is out of production, Grade 3 tumors are usually negative for ovalbumin because of their anaplastic nature, and tumors only in the ovary with no tumor in the oviduct may be ovalbumin positive. However, ovalbumin staining is the best single method for determining if an adenocarcinoma is of reproductive tract origin. Smooth muscle actin staining was useful for confirming leiomyomas and also for demonstrating that the mesenchymal tissue within adenocarcinomas often contains considerable smooth muscle. Technical difficulties in working with antibodies developed for human tissues to stain chicken tissue epitopes were encountered with several of the antibodies.
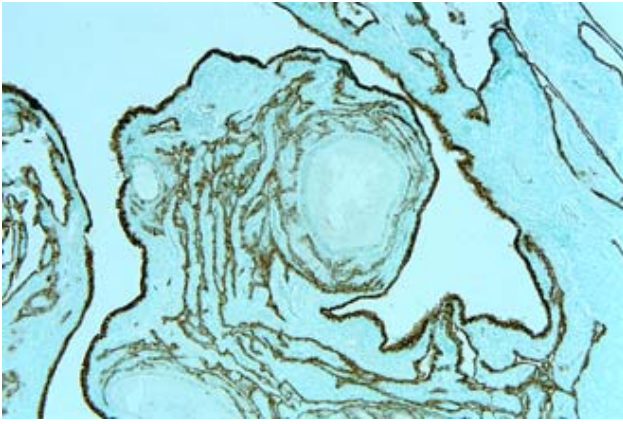
Figure 37. Immunohistochemical staining of normal ovary for pan-cytokeratin showing intense staining of the ovarian surface epithelium. Tumors showed similar staining. Granulosa cells in the follicles are not stained.
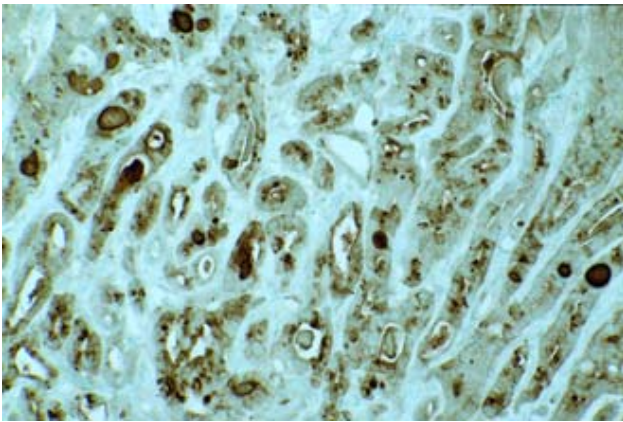
Figure 38. Adenocarcinomas in the ovary, oviduct, and metastatic sites are positive for ovalbumin unless the hen is not in production or tumor is anaplastic. Ovalbumin is not specific for oviductal adenocarcinomas but helps to identify a reproductive tract origin.

Key research accomplishments/Reportable Outcomes

-Establishment of large bio-repository of frozen and formalin fixed chicken reproductive tract cancers from birds of varying ages, strains, reproductive history and chemopreventive drug exposure
- Genetic analysis of chicken ovarian cancers for p53 and ras, with results similar to human
- Analysis of her 2/neu in chicken reproductive tract cancers, with results similar to human
- examination of TGF-beta surrogate biomarker showing an effect of progestin on   expression in chicken ovarian epithelium, results similar to human
- Basic techniques established for tissue processing and evaluation for histologic   characterization
- Development of a histologic criteria and characterization scheme for reproductive tract tumors in the chicken.

Summary/Conclusion: Ovarian Cancer in Laying Hens as an Animal Model

Progress in understanding ovarian cancer has been hampered by the lack of an animal model. We believe the chicken is an appropriate model for the disease and find it difficult to understand, when naturally occurring ovarian cancer is so common in chickens, that it is not being more fully exploited. We can only assume that the lack of interest in the chicken model is due to insufficient information, which provides the incentive for providing an overview in this report of the model based on our extensive work in this area. It is important to keep in mind that few, if any, animal models are exact

duplicates of the disease to be studied. The   question to be answered is, "How similar or different is ovarian cancer in laying hens to the disease in women?"

There are a number of very obvious and significant anatomical and physiological differences between reproduction in laying hens and women, even though the end product, production of viable offspring is the same. Birds have evolved numerous structural and functional modifications to permit them to fly. Quite simply, the biological strategy of the bird to preserve flight has been to minimize mass (weight) and maximize function. This has meant that as far as reproduction goes, the only parts of the reproductive tract in the normal laying hen that remain are the left oviduct and ovary. There is no uterus; embryo development occurs externally within a shelled egg. Thus, while the basic function of the mammalian reproductive tract is to nuture the development of the fetus, that of the bird is to secrete everything the embryo will need during the period it is developing externally. Most of the differences between ovarian cancers in birds and women can be readily explained by this fundamental difference in function – secretion vs. nuturing. The following are some key differences in the anatomy, physiology, and pathology of birds.

Anatomy
- Hens only have a single ovary and oviduct. The functional ovary and oviduct are located on the left side.
- Ovary is a diffuse structure with poorly defined medullary and cortical areas.
- Ovarian follicles develop on a stalk and not within the ovarian cortex.
- Oviduct is greatly enlarged.
- Extensive glandular tissue is present in the oviductal mucosa, especially magnum where the albumin that surrounds the ovum within the egg is produced.
- Specialized structures ("sperm-host glands") maintain viability of sperm for extended periods of time.
- Ovary and oviduct are located within a separate peritoneal (intestinal) cavity that occasionally communicates with the left dorsal hepatic cavity.

Physiology
- Avian reproductive tract is primarily secretory.
  - Ovum enlarges during a 7-11 day period after initiation (approx 100x volume, 200x wt); 19 g of yolk (65% lipoprotein) produced by liver daily (approx. 2g/follicle/day).
  - Oviduct secretes 32 g of albumen and 2-2.5 g calcium for each egg.
- Ovulation cycle is slightly less than one egg/day (25-28 hrs).
- 260 eggs are produced in 50 wks (equivalent ovulations of a woman over 20 yrs), hens typically lay two cycles over a 2-year period (exceeds a woman's life time ovulations).
- Less epithelial damage during ovulation.
- Progesterone at physiologic levels increases ovulations.
  - Receptors in hypothalamus, pituitary, ovary, and oviduct.
- Molting results causes involution and results in a renewal of the reproductive tract; risk of developing cancer is reduced.
- High lipid metabolism and daily loss through egg production.

Pathology
- Common occurrence of oviductal adenocarcinoma (relationship to ovarian adenocarcinomas is uncertain).
- Ovarian adenocarcinomas typically are composed of cells with oviductal glandular epithelial characteristics irrespective of presence or absence of an identifiable oviductal tumor.

- Tumor cells produce albumin in response   to hormonal signals that control albumin production in normal glandular epithelium.

Ovarian cancers in laying hens and women share a number of common features, which contribute to the potential usefulness of the chicken as a model of ovarian cancer. Among these are:

Disease
- Occurs spontaneously; does not need to be artificially induced by genetic manipulation, exposure to toxins, or infection with infectious agents.
- Incidence increases with age (33 – 40% at 4-yrs of age in ovulatory hens).
- Cancer rates vary with breed (strain) suggesting possible genetic susceptibility factors.
  - o Lower inhibin levels in strain with increased ovarian cancer (Johnson *et al.* 2005)
- Factors reducing risk of developing cancer include:
  - o Decreased ovulation.
  - o Diet restriction.
  - o Progestins in anovulatory hens.
- Restricting caloric intake to approximately 55% normal between 2 and 4 years of age resulted in decreased total mortality and cancers, especially those of the reproductive tract. Egg production was also sharply reduced (unpublished data).
- Affected hens are clinically normal and continue to ovulate until ovarian cancer is advanced.
- Disease is progressive and fatal. Advanced cases in both hens and women characterized by muscle depletion and marked ascites.

Pathology
- Epithelial adenocarcinoma; ovarian tumors in other putative animals not of epithelial origin.
- Scirrhous nature due to extensive fibrous and smooth muscle proliferation.
- Ascites and carcinomatosis from generalized peritoneal implantation and intravascular dissemination common. Ovarian cancer in the chicken is the only model that mimics this important feature of the disease in women.
- Patterns of metastasis are similar: peritoneal, intestinal (with potential obstruction), liver, lung. Chicken has greater relative involvement of the pancreas.
- Typical cells have oviductal epithelial characteristics consistent with Müllerian duct differentiation.
- Similar cell markers (Rodriguez-Burford *et al.*, 2001)
- *p*53 mutations are present in hot spots similar to human ovarian cancer.
- *ras* mutations are not present, similar to human ovarian cancer.
- Her2-neu expression in proportion of chicken tumors, and associated with cases Advanced disease, similar to human ovarian cancer.

Laying hens are readily available and are inexpensive domesticated laboratory animals. Hens normally complete their productive period at around 2 years of age when they are removed from the laying house. Currently they have no commercial value and are euthanized. The laying hen model would be ideal for screening preventive programs, examining the impact of nutrition on cancer development, and determining efficacy of treatments (other than lipid soluble compounds as these are deposited in the egg and lost). More is known about the nutritional needs of the chicken than any other animal including us and they readily lend themselves to nutritional interventions. We feel the chicken lends itself to basic studies on pathogenesis including histogenesis, tumor dissemination, and mechanisms of tumor implantation and growth. The chicken genome has been sequenced and they

are known to carry genes that impact the   occurrence of cancer including BRCA1 and BRCA2 genes (Orelli *et al*, 2001; Takata *et al*, 2002). Unfortunately, the chicken, because of its mode of reproduction does not readily lend itself to gene manipulation, but that is of limited importance since a high incidence of ovarian cancer occurs spontaneously. Also, reagents available for molecular study are limited for the chicken, but if the model were more widely used, it is anticipated that these would be developed and become available for use in basic studies. In conclusion, we believe the chicken model of ovarian cancer has considerable value for comparative study of the disease in women and that it should be more widely considered and used.

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Cliby, Rodriguez, Gonzalez, Berchuck, Whitaker, Lancaster, Wenham, Barnes, Carver, Anderson, Petitte. Genomic patterns in reproductive tract tumors in the chicken. Presented at Society of Gynecologic Oncologists Meeting 2005

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Windham J, Carver D, Rodriguez G, Robboy SJ, Barnes HJ. The anovulatory chicken: A model for testing whether chemopreventives can reduce rates of spontaneous ovarian cancer in the chicken. Merck-Merial National Scholar Symposium, Purdue Univ, W. Lafayette, IN, Aug 1-3, 2002.

* Note, multiple manuscripts are undergoing preparation.

Table 1. Classification of Avian Reproductive   Tract Neoplasia

_____

A.1 Normal tissue, artifact, inflammation, or other non-neoplastic lesion – **Report**
A.2 Proliferative tissue characteristic of neoplasia – **B**

   B.1 Mesenchymal cells – **C**
   B.2 Stromal infiltration and replacement by neoplastic blood cells - **D**
   B.3 Epithelial cells, cytokeratin positive, usually ovalbumin positive – **E**
   B.4 Smooth muscle with embedded neoplastic epithelium – **F**
   B.5 Multiple tissue types without defined organization – **Teratoma**
   B.6 Multiple tissues types with primitive renal differentiation - **Nephroblastoma**
   B.7 Tubules containing Sertoli cells and/or seminiferous cells –
        Sertoli, Seminiferous, Mixed tumor types
   B.8 Follicular pattern, individual and small groups of large cells, fine stroma –
        Granulosa cell tumor
        ▪ Well differentiated, localized or multifocal in ovary – Benign
        ▪ Poorly differentiated, multiple tissues affected – Malignant

   C.1 Well-differentiated smooth muscle cells – **Leiomyoma**
        ▪ Ventral mesosalpinx margin
        ▪ Tunica muscularis of oviduct or intestine, tunica media of large arteries
        ▪ Ovarian medulla
   C.2 Poorly differentiated, proliferating smooth muscle cells, mitotic figures, tissue invasion –
        Leiomyosarcoma
   C.3 Well-differentiated fibrocytes, collagen production – **Fibroma**
   C.4 Poorly differentiated, proliferating fibroblasts, tissue invasion, collagen production –
        Fibrosarcoma

   D.1 Infiltrates of lymphocytes or lymphoma
        ▪ Pleomorphic, T-lymphocytes, may affect immature females – **Marek's disease**
        ▪ Monomorphic, B-lymphocytes – **Lymphoid leukosis**
   D.2 Immature myeloid cells – **Myeloid leukosis**

   E.1 Well-differentiated, expanding, non-invasive, often polypoid – **Adenoma**
        ▪ Oviductal – glandular epithelium, cytoplasmic albumin granules, non-ciliated
        ▪ Infundibular cystadenoma – lining epithelium, ciliated
        ▪ Adrenal cortical tumor – left adrenal gland embedded in ovarian medulla
   E.2 Invasive, proliferating, multiple organs affected – **Adenocarcinoma**
        ▪ Differentiation
             o Grade 1: well-differentiated – mitosis rare to absent, defined pattern
             o Grade 2: mitosis rare to occasional, architectural pleomorphism but tubular pattern present,
                minimal to moderate cellular dysplasia
             o Grade 3: mitosis common, cellular pleomorphism, often in sheets, anaplasia
        ▪ Architecture
             o Tubular
             o Cystic
             o Papillary
             o Combinations of above
             o Lobular
             o Solid
        ▪ Oviductal – cytoplasmic granules, ovalbumin positive
        ▪ Ovarian adenocarcinoma
             o Type 1: cortical, expanding nodules, <25% (metastatic, secondary)
             o Type 2: medullary/cortical, infiltrating lobules, >25<75% (probable primary)
             o Type 3: medullary & cortical, >75% organ involved (primary)
        ▪ Morphologic Types
             o Albuminous (oviductal cell type)
             o Squamous differentiation
        ▪ Peritoneal – absence of oviductal tumor, absent or Type 1 ovarian tumor
        ▪ Metastatic tumor from other primary site – pancreas, intestine, kidney, other
        ▪ Metastatic from reproductive tract – serosal surfaces, liver, pancreas, lung, spleen, heart, kidney, muscle

   F.1 Well differentiated leiomyoma with <10% of tumor composed of embedded neoplastic epithelium –
        Leiomyocarcinoma

Appendix 1.

Case study set of representative normal reproductive tissues and epithelial tumors for immunohistochemical studies.

OVCA IHC Set

		H&E --					
		CA	OV	Type	OVD	Both	Comments:
35	1	0	0	0	*	0	Normal ovary - 2 sections
88	1	0	0	0	*	0	Normal ovary [oviduct cyst at hilus)
88	2	0	*	*	0	0	Normal oviduct - out of production
93	1	0	0	0	0	0	Normal ovary & oviduct - out of production
95	1	0	0	0	0	0	Normal ovary & oviduct - out of production
103	1	0	0	0	*	0	Normal ovary
103	2	0	*	*	0	0	Normal oviduct - out of production
119	1	1	1	3	*	1	Moderate variability among tumor foci
119	2	1	*	*	1	1	Extensive Ovd tumor, nml liver & thyroid
132	2	1	*	*	1	0	Early CA, multiple leiomyomas
142	1	0	0	0	*	0	Normal ovary
142	2	0	*	*	0	0	Normal oviduct - in production
160	1	0	0	0	0	0	Normal ovary & oviduct - out of production
161	1	0	0	0	*	0	Normal ovary
161	2	0	*	*	0	0	Normal oviduct - shell gland
171	2	0	*	*	1	0	Oviduct - leiomyoma, leiomyocarcinoma
230	1	0	0	0	*	0	Normal ovary
230	2	0	*	*	0	0	Normal oviduct - out of production
240	1	0	0	0	0	0	Normal ovary & oviduct - out of production
263	1	1	0	0	1	0	OVD cystadenocarcinoma (nice section)
264	1	1	1	3	*	1	Only ovary in set, oviduct positive on another slides
277	1	1	0	0	1	0	Extensive Ovd tumor, normal liver & ovary
302	1	1	1	3	0	0	Excellent ovarian tumor, normal oviduct on other slides
325	1	1	1	3	*	1	Only ovary in set, oviduct positive on other slides
330	1	1	1	1	*	1	OVCA Type 1, also oviductal tumor
330	2	1	*	*	1	1	Oviductal tumor, also OVCA Type 1
340	1	1	1	3	1	1	Extensive lesions, inflammatory reaction in ovary
410	1	1	0	0	1	0	OVD cancer, normal ovary & liver
415	2	1	*	*	1	0	OVD cancer, normal ovary on another slide, hematoma
419	1	1	*	*	1	0	OVD cancer, adenoma-CA transition, normal ovary
422	1	1	1	1	0	0	Primary OVCA – early
422	2	1	1	1	0	0	
441	1	1	0	0	1	0	V. early tumor
444	1	1	1	1	*	*	
481	1	1	1	3	*	1	Excellent advanced ovarian tumor, also kidney & thyroid
481	2	1	*	*	1	1	Multiple OVD CA, v. large leiomyocarcinoma, leiomyoma
512	2	1	*	*	1	1	Excellent early lesions, ovary on slide 1 negative
514	1	1	1	3	1	1	Very large tumors of ovary and oviduct
527	1	1	1	3	*	0	CystadenoCA of Ovary
563	1	1	1	3	*	0	Also serosa with confluent cancer on slide
586	1	1	*	*	1	0	OVD CA - early
702	1	1	1	3	0	0	
850	1	1	1	1	*	1	
850	2	1	*	*	1	1	Multiple OVD CA, out of production

875	3	1	1	1	*	0	
875	4	0	0	0	0	0	Oviduct infundibulum - cystadenoma
995	1	1	1	3	0	0	Excellent advanced ovarian tumor
1017	1	1	1	3	*	0	Excellent advanced ovarian tumor, lg tumor nodule
1039	1	1	1	3	*	0	Excellent advanced ovarian tumor
1061	1	1	1	3	*	0	Excellent advanced ovarian tumor
1095	1	1	1	2	*	1	
1095	2	1	1	2	1	1	

* tissue not on slide

CA = cancer
OV = ovary
OVCA = ovarian carcinoma
OVD = oviduct
Nml = no microscopic lesions

Note: please see also Appemdix 2 which contains a published manuscript.